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APPLICATION NUMBER: ***60/490,349***
FILING DATE: ***July 24, 2003***
RELATED PCT APPLICATION NUMBER: ***PCT/US04/24015***

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07/24/03 17607 U.S. PTO
16424 U.S. PTO 60/490349 07/24/03

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a r qu st f r filing a PROVISIONAL APPLICATION FOR PATENT und r 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

ENHANCED STEEP WATER

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 23
☐ Drawing(s) *Number of Sheets*
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☒ Other (specify) Return-receipt postcard

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Respectfully submitted,

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Date 7/24/2003

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REGISTRATION NO. *(if appropriate)* 43,581

Docket Number: CGL03/0260

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Serial No.	Filing Date 24 July 2003	Examiner	Group Art Unit
Invention: ENHANCED STEEP WATER			

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P06B/REV02

ENHANCED STEEP WATER

Field of the Invention

The present invention relates to the fermentation of steep water which comes from the corn wet milling process to produce a variety of enhanced steep water products.

Background

Corn wet milling is the processing of corn to make corn starch, and other corn products. Corn starch can be utilized *as is,* or subsequently processed into carbohydrate and protein feedstocks. Corn kernels are sequentially steeped, and then milled and separated into their major constituent fractions. The "solubles" fraction, or light steep water, is a product of steeping the corn, while the germ, fiber, starch, and protein fractions are products of the milling step. Steep water provides a relatively inexpensive starting material that includes a number of nutrients that are utilized as an ingredient in animal feed and fermentation applications.

Summary

The present invention is directed to methods of making and using enhanced steep water products, as well as the products themselves. These methods utilize steep water, a co-product of corn wet milling. These enhanced steep water products may be used as additives in animal feed to enhance productivity and/or reduce the cost of production, or as nutrient inputs for subsequent fermentation processes. Examples of enhanced steep water products include steep water with increased levels of bacteriocins, vitamins, yeast and yeast extracts, enzymes, amino acids, organic acids, direct-fed microbials and combinations thereof.

The enhanced steep water products described herein can be made through a variety of methods, such as by altering the conditions in the steep water to create an environment that will allow specific populations of microorganisms to grow. These are referred to as either endogenous (naturally present in the steep water) or exogenous (added to the steep water). The microorganisms can be fungal, yeast, bacterial, or combinations thereof depending on the desired enhanced steep water product.

The enhanced steep water products can be used as a "feedstock" input in subsequent fermentations, or blended into other matemals such as animal feed.

In one aspect, endogenous yeast and/or supplemental yeast are grown in light steep water or concentrated light steep water to obtain yeast biomass steep water. Thereafter, the biomass may be hydrolyzed to obtain a hydrolyzed yeast biomass. The biomass or hydrolyzed biomass may be blended into animal feed as an additive to improve animal productivity, or used as a "feedstock" nutrient for subsequent fermentation processes.

During the yeast growth phase, the majority of reducing sugars and lactate are consumed for biomass and/or glycerol production. Low sugar and low lactate yeast-steep water is useful for fermentation processes that are sensitive to lactic acid. High glycerol containing yeast-steep water would have the advantage over conventional steep water for its freeze-thaw stability and sweetness. Glycerol is also useful for many pharmaceutical fermentation processes.

Light or concentrated light steep water also may be fermented with microorganisms, such as *Lactococcus lactus* under fermentation conditions of temperature, time, pH and aeration effective to produce bacteriocins. Endogenous microbes in light steep water can also be induced to produce bacteriocins. The bacteriocin fermentation product may be included with an animal feed to improve animal productivity.

In another aspect of the invention, light or concentrated light steep water is fermented with microorganisms under fermentation conditions of temperature, time, pH and aeration effective to produce lysine and methionone.

According to the invention, light or concentrated light steep water is fermented with microorganisms under fermentation conditions of temperature, time, pH and aeration effective to produce direct –fed microbials (DFM) that are useful as a nutrient supplement in animal feed. After fermentation, at least some of the DFM are separated from the fermented steep water, prior to concentration of the steep water by evaporation, followed by addition of the separated DFM into the concentrated steep water product.

Finally in this aspect of the invention, light or concentrated light steep water is fermented with microorganisms under fermentation conditions of temperature, time, pH and aeration effective to produce vitamins, such as vitamin B_{12}, riboflavin, arachidonic acid, dihomo-gama-lineolenic acid, thiamine, pathotenate, and mead acid, or vitamin precursors, such as the vitamin C precursor 5-ketogluconic acid.

In another aspect of the invention the corn steep liquor product starting material may be the fermentation product of light or concentrated light steep water. In this aspect the light or concentrated light steep water is fermented with lactic and/or propionic acid producing bacteria to provide an acid enriched steep water. The steep water is fermented at a time and temperature to decrease the pH of the steep water to less than about 5, preferably to a pH in the range of about 3.8 to about 4.6. The fermentation may be conducted with endogenous bacteria, with added lactic acid bacteria, and/or propionate producing bacteria. The fermentation is important because it converts residual sugar in the steep water into organic acid, such as, for example, lactic acid, which lowers the pH of the steep water and thereby enhances the stability of the steep water. Low steep water pH also increases the solubility of steep solids and minimizes precipitate formation during the evaporation process of corn steep liquor. The fermentation also is effective for reducing the sugar content in the steep water in an amount such that browning reactions do not deleteriously affect the color of any feed to which the acid enhanced product may be added as a result of drying the feed.

In yet another aspect of the invention, the steep water product starting material is light or heavy low phosphorous steep water which has a phosphorous content which is not more than about 25 weight percent of the steep water from which the low phosphorous steep water has been made and which has not been reduced in phosphorous content. Corn gluten feed is primarily used for cattle feeding and has about four times the amount of phosphorus needed by animals for nutrition. Moreover, much of the phosphorus is in the undesirable form of phytate [mysoinsoitol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)].

In wet milling of corn for corn starch, kernel residues remain that include corn germ, corn bran, insoluble protein, and corn solubles. The wet milling of corn includes steeping of the corn prior to breaking the corn. Most of the phosphorus in corn is in the form of an organic phosphorus containing compound, phytate. Steeping among other things leaches phytate out of the corn into steep water and ideally the steep water is used as part of the animal feed once it is evaporated to about 50% solids to form heavy steep water or corn steep liquour. Corn steep liquor is also used as a nutrient source for various fermentation processes.

Phytate is poorly digested by monogastric animals. Ruminants, such as cattle, can digest phytate through microorganisms found in the gastrointestinal tract and hence utilize released phosphate, but excess dietary phytate and phosphate consumed by a ruminant animal will pass

through its gastrointestinal tract, be excreted as manure and become environmentally damaging in areas of extensive livestock production. This is because excessive amounts of phosphorus enter the environment and the aquifer from the animal manure. A further problem with phytate is that it associates with multivalent cations which may be nutritionally needed by the animal, and thus interfere with the bio-availability of these cations to the animal.

The phosphorus reduced aspect of the invention contemplates using steep water from wet corn milling and removing the phytate from the steep water by mixing the steep water with an alkaline hydroxide, such as calcium hydroxide, magnesium hydroxide, ammonium hydroxide and mixtures thereof, to convert the phytate to an alkaline metal salt and/or ammonium salt (phytin) and to precipitate the phytin in the steep water to provide a phytin precipitated steep water. The alkaline metal and/or ammonium hydroxide is in an amount effective to precipitate the phytate in the steep water and to provide an alkaline metal or ammonium phytin complex or associate the divalent metal and/or ammonium ion with the phytin such that the phytin will precipitate with the calcium metal, magnesium metal and/or ammonium ions. Calcium ions, however, are a very important aspect of the invention and work better to precipitate phosphorus than other ions even when the other ions are in an environment having a high pH. The alkaline metal or ammonium ions also complex and precipitate a small amount of inorganic phosphate in steep water. Generally the alkaline metal and/or ammonium hydroxide will be present in amount to provide a pH of greater than about 5.5 and preferably greater than about 6.0, and a Ca/P molar ratio which is effective to precipitate at least 75% and preferably 80% of the phosphorus, which ratio is at least about 1.0, preferably at least about 1.2. Thereafter the ion/phytin complex is separated from the steep water to provide a low phosphorus steep water. After separation of the precipitated phytin from the steep water, the low phosphorus steep water is used as a starting material for fermentations according to the invention.

The low phosphorous steep water may be used a starting material for the fermentations as described above to produce organic acids, vitamins, bacteriocins, enzymes, lysine and methionine, probiotic or direct-fed microbial supplements, yeast and yeast extract, and products high in free amino acid nitrogen content. Each of the latter fermentation products then may be mixed with or used as an animal feed with a low phytate content.

Finally according to the invention, the steep water product starting material may be the low phytate steep water, which has been fermented with microorganisms, which produce lactic

acid and/or propionic acid. This fermentation produces a low phytate acid enhanced steep water product starting material. The low phytate acid enhanced starting material then may be fermented as described above with yeast, lysine and methionine forming bacteria, vitamin forming bacteria, bacteriocin forming bacteria, and free amino acid nitrogen forming bacteria. These fermentations produce low phytate acid enhanced products which, depending on the fermentation, includes a yeast biomass or an extract of yeast, organic acids, lysine and methionine, one or more vitamin(s), one or more bacteriocins, one or more enzymes, and free amino acid products. The low phytate acid enhanced lysine and methionine product, the low phytate and enhanced yeast biomass, and the low phytate acid enhanced enzyme product may be used as or mixed with an animal feed. The low phytate acid enhanced yeast biomass also may be hydrolyzed and then used as or mixed with another animal feed.

Detailed Description

I. Definitions

"Phytate" means myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate. This compound associates with cations and forms complexes that are sometimes called phytin. We shall also describe these metal or ammonium ion/phytate associated molecules as phytin complexes.

"Corn gluten feed" is a co-product of the wet milling of corn for products such as corn starch and corn syrup. Corn gluten feed generally includes corn germ, corn bran, corn solubles, cracked corn, and fermentation end products.

"Steep water" includes all varieties of water that are removed from the corn after steeping. For example, one variety of steep water is "light steep water" which contains the soluble materials (including protein, amino acids, sugars, and phytate) originating from the corn kernel and fermentation products (mainly lactic acid and ethanol) produced from the fermentation of corn solubles during steeping. Typical light steep water has a dry solids content of about 8 - 12%. Another variety of steep water is "heavy steep water" which is steep water that has a dry solid content of about 50%. Finally "concentrated steep water" is steep water that has from about 12% dry solids to about 49% dry solids.

"Reducing Agent" means the sum of gaseous sulfur dioxide, sulfurous acid, bisulfite ions, and sulfite ions, in the steeping process.

II. Making Enhanced Steep Water

A. Steeping Generally

The first step in the corn wet milling process is cleaning the corn. Bulk corn is cleaned on vibrating screens to remove coarse material and fine material. These screenings remove solid material from the corn kernels so that the material does no cause processing problems such as restricted water flow through the steeping process and increased steep water viscosity.

The second step in the corn wet milling process is steeping which is the soaking of the corn in water under controlled processing conditions of temperature, time, and reducing agent concentration. During the steeping process a fermentation also occurs because the naturally occurring population of microorganisms grow. These populations contain bacteria such as lactic acid bacteria, yeast, and fungus. The combination of environmental conditions and the resulting fermentation have been found necessary to promote diffusion of the water through the tip cap of the corn kernel into the germ and endosperm. This facilitates the softening of the kernels, which allows for better separation of the components of corn.

Steeping is accomplished by putting corn into tanks and covering the corn with water. The corn and water blend is heated to about 52°C (125°F) and held for about 22 to about 50 hours. Steeping may be done by continuously adding dry corn at the top of the steep while continuously withdrawing steeped corn from the bottom.

Water from the steeping process accumulates corn solubles. The water is treated with a reducing agent such as sulfur dioxide to a concentration of about 0.12 to about 0.20 weight percent. The sulfur dioxide increases the rate of water diffusion into the kernel and assists in breaking down the protein-starch matrix, which is necessary for high starch yield and quality.

As water moves from one steep tank to another it is advanced from steep to steep, and the sulfur dioxide content decreases and bacterial action increases. This results in the growth of lactic acid bacteria. The lactic acid concentration is usually about 16 to about 20% (dry basis), and the sulfur dioxide (content drops to about 0.01% or less) after the water has advanced through the steeping system.

This light steep water contains the solubles soaked out of the corn as well as products from endogenous microbial fermentation.

Optionally, steep water can be treated to remove phosphorous using any method known in the art, such as by mixing the steep water with $Ca(OH)_2$ and/or $Mg(OH)_2$ to precipitate the phytate in the steep water. Precipitation of light steep water containing solids, and phytate, that

has a pH of about 4 is accomplished by mixing a sufficient amount alkaline metal hydroxide, such as lime, and/or ammonium hydroxide (at least about 0.07%, preferably about 0.07 to about 3.0%, most preferably about 0.3 to about 1.0% w/w) to raise the pH of the light steep water to above about 5.5 and to precipitate at least about 75% of total phosphorus in steep water as phytin and insoluble phosphate, such as calcium phosphate. The method is also effective for precipitating at least about 80% of total oxalate in the steep water such as insoluble calcium oxalate. Generally, more than about 90% of phytate and about 10 to about 50% of inorganic phosphate are precipitated out of steep water as the calcium salt, and more than about 90% of the oxalate is precipitated out of steep water as calcium oxalate. The resulting steep water containing white calcium phytate/phosphate precipitate and calcium oxalate precipitate is subjected to vacuum filtration or horizontal basket centrifugation to produce a calcium phytate and calcium oxalate product and a low phosphorus steep water.

B. Altered Steep Water Useful for Making Enhanced Steep Water

Enhanced steep water products are made by fermentations that are conducted directly in the steep water. Accordingly, the fermentations can be conducted in a steep water that has enhanced levels of lactic and/or propionic acid, steep water with reduced phosphorus levels, and steep water with reduced phosphorous levels that also has enhanced levels of lactic and/or propionic acid. Steep water that can be used to make enhanced steep water are described as follows: steep water with enhanced lactic acid. (CSL+); corn steep water with a reduced phosphorus content (LPCSL); corn steep water with reduced phosphorus content which has been fermented with lactic acid bacteria (LPCSL+); and corn steep water which has been fermented with proprionic acid bacteria, corn steep water with reduced phosphorus content which has been fermented with propionic acid bacteria (HPCSL+).

Enhanced steep water can be generated by inoculating (i.e. exogenous microorganisms) into the water that is added to the corn at the any point in the steeping process. Thus, increasing the population of an endogenous microorganism or providing a microorganism that is not normally present in the steeping process. For example, the microorganism can be added when the concentration of the reducing agent becomes low enough to allow the microorganism population to increase.

Enhanced steep water can also be made by altering the conditions under which the steeping actually occurs. The altered conditions will allow exogenous or endogenous microorganisms to grow.

III. Uses of Enhanced Steep Water

The feeds with which the enhanced steep water products of the invention may be mixed include but are not limited to corn gluten feed, soy hulls, wheat middlings, and other cereal grain fibers, which are co-products from milling. In one embodiment, the feed ingredients are from corn and include corn bran, cracked corn, extracted cornmeal and distillers' solubles or corn processing co-products to make a high moisture corn gluten feed. Such a high moisture feed will contain from about 30 to about 70 weight percent moisture. Alternatively, the feed augmented with the enhanced steep water products may be mixed with the other fibrous feed components and then dried and pelletized to a dry feed such as a dry corn gluten feed.

Enhanced corn steep water products may be made such that they have a greater content of lactic acid, propionic acid, yeast, free amino acid nitrogen (FAN), bacteriocins, vitamins, enzymes, lysine, methionine, direct-fed microbial supplements and/or combinations thereof when compared to steepwater that has not been treated to enhance production of one or more of these products.

The resulting products may be incorporated into animal feeds or used as a component in fermentation media.

IV. Organic Acids Enhanced Steep Water

The preservation of animal feed ingredients/complete feed products during storage and transport is commonly achieved by addition of chemically-derived organic acids, such as propionic acid, or their salts. Propionic acid, a potent mold inhibitor, can also be generated via fermentation by a few microbial genera, such as *Propionibacterium*. Corn steep water, a rich source of protein and vitamins, is utilized as a component of animal feed and can serve as a fermentation feedstock for endogenous propionic acid-producing microorganisms, as well as exogenously added microbes. Consequently, fermentation of steep water by microorganisms to produce propionic acid creates a feed component with a natural preservative present that may provide economic advantage versus the addition of chemically-derived counterparts. Steep water with elevated propionic acid levels could be used as a feedstock for microbial fermentations

resistant to propionic acid, yet prone to fungal contamination. In addition, organic acids such as lactic, citric and fumaric acid are routinely added to animal feeds to reduce gastric pH, suppress pathogenic organisms and/or as readily available energy sources via the Krebs Cycle. Fermentation of steep water by organisms that would produce these acids might offer an economical, natural alternative to current practice. Finally augmenting an organic acid-rich steep water with additional desired acids to meet a desired commercial specification might also be possible.

Steep water can be inoculated with one or more microorganisms to produce one or more organic acids. Similarly steep water can be incubated under conditions to allow endogenous microorganism to grow and produce organic acids. Organic acids that may be useful include citric acid, propionic acid, lactic acid, succinic acid, and the like.

Steep water or a phosphorus reduced steep water may be fermented for a time and at a temperature which is effective to convert carbohydrates in the steep water into an organic acid such as lactic acid and/or propionic acid to provide an organic acid enhanced steep water. The organic acid enhanced steep water may be used as a nutrient source for other fermentations or combined with an animal feed to provide organic acid enhanced steep water containing animal feed.

For example, low phosphorus steep water may be fermented using the endogenous bacteria (or added lactic acid forming bacteria) at a temperature of at least about 45°C, preferably about 45 °C to about 55°C for at least about 8 hours, preferably about 8 to about 48 hours to convert fermentable sugars to lactic acid and to reduce the pH to less than about 5.0. The low pH and low phosphorus steep water is dried to about 30 to about 90% solids and mixed with other feed ingredients to make a high moisture corn gluten feed. The low pH and low phosphorus steep water containing feed is dried to about 6 to about 15 weight percent moisture to provide the phosphorus reduced corn gluten feed of the invention having less than about 25 weight percent phosphorus than a comparable corn gluten feed containing untreated steep water. This feed also may be pelletized. The pH stabilized, low phosphorus steep water can be used as is or can be dried to about 30 to about 90% solids and used as a fermentation nutrient feedstock or as light steep water. The pH stabilized, low phosphorus steep water will have a minimal impact on the mineral metabolism of the fermentation organisms.

Furthermore, the low phosphorous steep water can be evaporated to about 30 to about 90% solids and combined with other feedstuff to make a generic high moisture low phosphorous animal feed. The high moisture animal feed produced using the low phosphorous steep water which has been fermented to produce lactic acid and low pH has less mold formation after 5-14 days as compared to high moisture animal feed (more than about 12 weight percent moisture) produced with low phosphorus steep water or steep water that has not been fermented. Endogenous and/or added lactic acid bacteria may be utilized to produce lactic acid in the steep water.

Endogenous and/or propionic acid bacteria may be added to the steep water prior to fermentation. One example of propionic acid bacteria that may be utilized in the process is *Propionibacterium acidipropionici* strain ATCC 55737. A portion of the low phosphorous steep water may be fermented and then recombined with the remaining low phosphorus steep water. In this aspect of the invention, an amount of steep water is fermented such that when the fermented steep water is recombined with the remaining steep water and used to produce a high moisture animal feed, the feed has from about 1 to about 4 lbs. of propionate per ton of feed. The high moisture animal feed produced using low phosphorous steep water which has been fermented to produce propionate has less mold formation after 5-14 days as compared to an animal feed produced with low phosphorous steep water or steep water that has not been fermented to produce propionate. The fermented low phosphorous, high propionate containing steep water may also be held separately for other uses.

Lactic acid bacteria which may be used in the invention include *Lactobcillus spp., Lactococcus spp., Leuconostoc spp.* and *Bacillus coagulans*. Propionic acid bacteria are selected from the group consisting of *Propionibacterium acidipropionici, Propionibacterium freudenreichii, Propionibacterium shermanii, Propionibacterium jensenii*, and *Propionibacterium thoenii*. The method is effective for providing an organic acid enhanced steep water having at least about 20% lactic acid on a dry weight basis and/or at least 10% propionic acid on a dry weight basis. In an important aspect, the organic acid enhanced steep water includes physiologically suitable singlet organic acids and/or mixed acid constructs. The organic acid and mixed acid constructs are selected from the group consisting of formic acid, benzoic acid, lactic acid, propionic acid, acetic acid, citric acid, their salts, and mixtures thereof.

V. Yeast Enhanced Steep Water

Yeast and yeast extracts are another widely used nutrient source for animals and fermentation microorganisms. Yeast is also a rich source of vitamins and minerals such as pantothenate, biotin, thiamine, potassium, calcium, magnesium, iron, and zinc.

Corn steep water product may be fermented under controlled parameters to provide a yeast-enriched biomass. The yeast biomass may be used directly in animal feed or as a fermentation feedstock or may be hydrolyzed prior to further use. In accordance with the method of the invention, the steep water is fermented with endogenous yeast or with added yeast selected from the group consisting of *Saccharomyces cerevisiae, Candida utilis, Kluyveromyces marxianus* and *Torulaspora delbrueckii*. The fermentation is conducted at a temperature of at least about 28°C for at least about 48 hours.

VI. Bacteriocin Enhanced Steep Water

Antimicrobial metabolites including metabolites such as organic acids and bacteriocins provide a preservative function in foods and animal feeds. Bacteriocins are antimicrobial peptides produced by many strains of lactic acid and/or other bacteria used.

Bacteriocins generally exert their anti-microbial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing membrane pore formation, subsequently resulting in death. Accordingly, one aspect fo the invention provides methods of incorporating bacteriocins into foods by the direct addition of bacteriocin-producing cultures into steep water. The resulting bacteriocin enhanced steep water can then be added to food

Bacteriocin enhanced steep water can be fermented under controlled parameters that are effective for inducing elevated levels of bacteriocins from endogenous bacteria by adding exogenous bacteria to the steep water. Bacteriocins include compounds such as nicin, subtilisin and lactococcin. The bacteriocin enhanced steep water may be used directly in animal feed. More specifically, steep water may be fermented with endogenous bacteria or with added bacteria selected from the group consisting of *Lactobacillus spp., Lactococcus spp., Pediococcus spp., and Streptococcus spp.* Fermentation is conducted at a temperature within the range of 25°C to 60°C for 24-72 hours.

VII. Vitamin Enhanced Steep Water

Corn steep water can be fermented under controlled parameters to increase the amount of vitamin(s) in the steep water to provide a vitamin enhanced steep water product. The vitamin enhanced steep water product may be used directly in animal feed. In accordance with the method of the invention, steep water may be fermented with endogenous microorganisms or with exogenous microorganisms selected from the group consisting of *Propionibacterium shermannii, Ashbya gossypii, Eremothecium ashbyii, Bacillus spp.,Gluconobacter oxidans subsp. suboxidans, Serratia marcescens, Pseudomonas denitrificans, Mortierella alpina* and combinations thereof. Fermentation is conducted at a temperature of at least about 20°C for at least about 10 hours. The method is effective for providing a steep water having at least about 10 micrograms/L increased vitamin(s) content compared to the starting content of the vitamin(s) in the steep water product. Vitamins that can be produced include, but are not limited to vitamin B_{12}, riboflavin, vitamin C, biotin, and vitamin precursors such as the vitamin C precursor 5-ketogluconic acid.

VIII. Enzyme Enhanced Steep Water

Steep water may be used to ferment endogenous and/or exogenous microorganisms to produce vitamin enhanced steep water. Vitamin enhanced steep water can be produced using *Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus subtilis, Bacillus subtilis* containing a *Bacillus amyloliquefciens* gene for protease, *Aspergillus niger, Aspergillus oryzae, Bacillus lentus, Humicola insolens, Trichoderma longibrachiatum, Bacillus licheniformis, Bacillus licheniformis* containing a *Bacillus stearothermophilus* gene for α-amylase, *Bacillus stearothermophilus, Aspergillus niveus* and mixtures thereof. The microorganisms are cultured under fermentation conditions, such as temperature, pH, aeration and time which are effective to produce enzymes, such as proteases, xylanses, amylases and phytases and/or combinations thereof. Fermentation is conducted at a temperature of at least about 28°C for approximately 24-72 hours.

The enzyme enhanced steep water product may be used directly in animal feed or as a fermentation feedstock.

IX. Amino Acid Enhanced Steep Water

The amino acid content of animal feeds and fermentation media can limit the growth and/or productivity of animals and microorganisms. Amino acid enhanced steep water can be added to animal feed and/or fermentation media to increase the growth and/or productivity. Amino acid enhanced steep water can be made via several different methods. For

example, microorganisms that have been genetically engineered to over produce one or more amino acids or exogeneous microorganisms that over produce one or more amino acids can be added to steep water. Free amino acid concentration in steep water can also be increased by introducing organisms that degrade proteins to produce free amino acids.

Accordingly, steep water may be fermented under controlled conditions to provide an increased amount of free amino acid nitrogen (FAN), thus making an enhanced steep water. The FAN enhanced steep water product may be used directly in animal feed or as a fermentation feedstock. In one embodiment of the invention, steep water is fermented with endogenous bacteria or exogenous bacteria selected from the group consisting of *Bacillus subtilis, Bacillus amyloliquefaciens, Aspergillus niger, and Aspergillus oryzae*. Fermentation is conducted at a temperature of at least about 28°C for approximately 24-72 hours. The method is effective for providing an enhanced steep water product having at least about 4 mg/ml free amino acid nitrogen.

Current agricultural practices supplement lysine and methionine to animal feeds to provide optimal growth and feed utilization, which can be a costly process for producers. Thus, feed or feed components such as enhanced steep water, containing increased quantities of these limiting amino acids are particularly useful. Steep water with elevated lysine or methionine may also serve as enhanced fermentation feedstocks in that growth on these feedstocks allow for increased microbial growth.

Corn steep water can be fermented for a time and at a temperature that is effective to provide a steep water with enhanced levels of lysine and/or methionine as compared to steep water that has not been fermented. The lysine/methionine enhanced steep water product may be used as a fermentation feedstock for other fermentations, or added to an animal feed to enhance its nutritive content. In accordance with the method of the invention, steep water product may be fermented with endogenous bacteria or with exogenous bacteria that are effective for producing lysine and/or methionine such as ones selected from the group consisting of *Corynebacterium glutamicum, Corynebacterium acetoacidophilum, Brevibacterium lactofermentum, Brevibacterium flavum, Brevibacterium divaricatum,* and *Corynebacterium lilium*. Fermentation is conducted at a temperature of at least about 30°C for at least about 48 hours. The method is effective for providing steep water having at approximately 5 mg/ml lysine and/or approximately 1 mg/ml methionine.

X. Direct-Fed Microbial (Probiotic) Enhanced Steep Water

Steep water can be fermented for a time and at a temperature, which is effective to provide a steep water with enhanced levels of beneficial organisms that can be incorporated directly into foods, such as animal feeds. Direct-fed microbial (DFU) enhanced steep water may be used as a nutrient source for other fermentations or combined with an animal feed to provide a DPM enhanced steep water containing animal feed. In accordance with the method of the invention, steep water may be fermented with endogenous and/or exogenous microbes selected from the group consisting of *Lactobacillus plantrum, Lactobacillus casei, Lactobacillus acidophilus, Enterococcus faecium, Bifidobacterium bifidium, Bifidobacterium thermophilum, Bifidobacterium longum, Bacillus subtillis, Saccharomyces cerevisiae, Aspergillus oryzae* and mixture thereof. Fermentation is conducted at a temperature of at least about 25°C for at least about 10 hours. Thereafter, cells can be partially removed by centrifugation, the fermented steep water then concentrated by conventional steep water evaporation, followed by cooling to a temperature the specific microorganism can tolerate, then the removed cells are added back to the cooled concentrated product, to maintain the viability of the DFM organisms which would have otherwise in most cases been killed during evaporation.

The following examples illustrate methods for carrying out the invention and should be understood to be illustrative of, but not limiting upon, the scope of the invention, which is defined, in the appended claims.

EXAMPLES

BACETERIOCIN EXAMPLES

Example 1. Enrichment of bacteriocin producers in steep water

Lactic acid bacteria and other bacteria may be enriched, or exogenously applied and/or enriched by incubating light or concentrated steep water samples at various temperatures such as room temperature, 30°C, 37°C and/or 42°C, to encourage the growth of specific bacteriocin

producing bacteria (Contreras et. al., 1997 – Appl Environ Microbiol, Jan,63(1):13-20). Factors such as temperature, pH, water activity, redox potential and the presence of inhibitory compounds in the various corn steep water samples will determine bacterial growth. The bacteriocins produced may be identified using established analytical procedures. In addition steep water samples may be supplemented with nutrients to support the growth of the bacteriocin producing microorganism. Depending on the incubation times being employed, nutrient depletion will be compensated for by the addition of fresh medium. Limitation of specific essential molecules needed for cell metabolism, and amino acids, vitamins, minerals etc, may decrease the growth rate. Fed-batch fermentation is a viable method to obtain high concentrations of industrially significant bacteriocins from bacteria.

Example 2. Combination of propionic acid and bacteriocins

Similar to example 1, except low phosphorous steep water (LPCSL) will be used to enrich for propionic acid bacteria instead of lactic acid bacteria. The propionic acid bacteria produce some bacteriocins and are useful in preventing spoilage in dairy products and other foods. The elevated levels of propionic acid would be effective in preventing fungal growth in grains, non-grain feed ingredients, complete feeds and feed products. Steep water with increased propionic acid content as well as bacteriocins will have the benefits of bacteriocin as well as anti-fungal properties conferred by propionic acid.

VITAMIN EXAMPLES

Example 1. Fermentation with Endogenous Microorganisms to Produce Vitamin B_{12}

Low phosphorous light steep water (pH 6.0) is incubated at 32°C for 144h to allow for growth of endogenous microorganisms that produce propionic acid and vitamin B_{12}. Approximately 15 g/L lactate, 10 g/L dextrose and 5 g/L fructose are converted to propionic acid at yields near 50% of the initial substrate concentrations and to 5mg/L vitamin B_{12}. The resulting steep water with increased B_{12} is blended with other feed ingredients to make an animal feed

enriched with vitamin B_{12}, or alternatively used as a fermentation feedstock for a B_{12} requiring fermentation.

Example 2: Fermentation with *Propionibacterium shermannii* to Produce Vitamin B_{12}

Low phosphorous light steep water (pH 6.0) is inoculated with *Propionibacterium shermannii* and incubated at 32°C for 144h. Approximately 15 g/L lactate, 10 g/L dextrose and 5 g/L fructose are converted to propionic acid at yields near 50% of the initial substrate concentrations and to 5mg/L vitamin B_{12}. The resulting steep water with increased B_{12} is blended with other feed ingredients to make an animal feed enriched with vitamin B_{12}, or alternatively used as a fermentation feedstock for a B_{12} requiring fermentation.

ENZYME ENRICHED CORN STEEP EXAMPLES

Example 1. Fermentation with Indigenous Microorganisms

Steep water or low phytate steep water is incubated at 30-40°C and neutral pH with aeration for 48-120 h to allow the indigenous *Bacillus* spp. to grow. The pH is maintained near neutral by buffering with phosphates or calcium carbonate. At end fermentation, the corn steep water's suspended solids, free carbohydrate, and free amino acid concentrations are reduced with the concomitant production of protease. A typical concentration of enzyme protein is 1-5% of the initial dry matter and cell biomass of 2-10%. At the end of the fermentation, the enzyme-enriched corn steep water is rapidly cooled to 4°C or evaporated to the desired solids content. Protease activity in the enzyme-enriched corn steep water is assayed using established analytical procedures.

Example 2: Fermentation with Bacillus

Same as Example 1 but rather than relying solely on the indigenous *Bacilllus* population, the steep water is inoculated with *Bacillus* spp. to produce protease.

Example 3: Fermentation with Aspergillus

Same as example #1 but the steep water is incubated at 30-35°C to allow the indigenous *Aspergillus* spp. to grow, or alternately inoculated with exogenous *Aspergillus* spp. In the latter instance, very high aeration and agitation is necessary due to the viscosity imparted by the growing fungal mycelium. Phytase activity in the enzyme-enriched corn steep water is assayed using established analytical procedures.

DIRECT-FED MICROORGANISM EXAMPLES

Example 1: Incubation with Endogenous or Added Yeast

Low phosphorus steep water is incubated at 25°C for 48 hours with aeration with either endogenous yeasts or added yeasts such as *Saccharomyces cerevisiae*. Approximately 10 g/L glucose, 5 g/L fructose, and 15 g/L lactate are converted to yeast biomass and CO_2. This yeast biomass is largely removed by centrifugation, the remaining water concentrated by evaporation to 50% dry solids, then cooled to 25°C, then the biomass harvested by centrifugation is added to the concentrated corn steep liquor product enriched with viable yeast. The product then may be mixed with an animal feed to form a component thereof.

Example 2: Incubation with Endogenous or added Lactic Acid Bacteria

Low phosphorus steep water is incubated at 37°C for 48 hours with either endogenous lactic acid bacteria (LAB) or added LAB such as *Lactobacillus acidophilus*. Approximately 10 g/L glucose and 5 g/L fructose are converted to a lactic acid (LAB) biomass and lactic acid. This LAB biomass is largely removed by centrifugation, the remaining water concentrated by evaporation to 50% dry solids, then cooled to 37°C, then the biomass harvested by centrifugation is added to the concentrated corn steep liquor product enriched with viable LAB. The product is used as a component of an animal feed.

Example 3: Incubation at 42°C

The process according to Example 2 except the low phosphorus steep water is incubated at 42°C. The product is used as a component of an animal feed.

Example 4: Incubation at 47°C

The process according to Example 2 except the low phosphorus steep water is incubated at 47°C. The product is used as a component of an animal feed.

LYSINE AND METHIONINE-ENRICHED STEEP WATER EXAMPLES

Example 1. Incubation with Endogenous Lysine Producers

Low phosphorous steep water at pH 7 is incubated at 30°C for 48 hours to allow endogenous lysine-producers to grow. Steep water is agitated at a rate of 150 rpm and dissolved oxygen concentration is maintained at 40% saturation. Biotin is added at a final concentration of 20 g/L to supplement growth. Lysine concentrations are monitored spectrophotometrically. After fermentation completion, the resulting steep water with increased lysine content is evaporated and blended with other feed ingredients to make an animal feed enriched in lysine. In addition, the steep water with increased lysine content can be used as an enhanced feedstock for fermentations.

Example 2. Incubation with Endogenous Methionine-Producers

Same as example 1 except low phosphorous steep water is incubated at 30°C for 48 hours to allow endogenous methionine-producers to grow.

Example 3. Incubation with Lysine-Overproducing Mutants of *Corynebacterium* or *Brevibacterium* spp.

Same as example 1 except low phosphorous steep water is inoculated with lysine-overproducing mutants of *Corynebacterium* or *Brevibacterium* spp.

Example 4. Incubation with Methionine-Overproducing Mutants of *Corynebacterium* or *Brevibacterium* spp.

Same as example 1 except low phosphorous steep water is inoculated with methionine-overproducing mutants of *Corynebacterium* or *Brevibacterium* spp.

STEEP WATER WITH PROPIONIC ACID EXAMPLES

Example 1. Fermentation with Endogenous Microorganisms to Produce Propionic Acid

Low phosphorous light steep water (pH 6.0) is incubated at 32°C for 72 h to allow for growth of endogenous microorganisms that produce propionic acid. Approximately 15 g/L lactate, 10 g/L dextrose and 5 g/L fructose are converted to propionic acid at yields near 50% of the initial substrate concentrations. Other acids such as acetic acid and succinic acid are also produced at approximately 25% and 5% of the initial fermentable substrate concentration, respectively. Determination of organic acid concentrations is accomplished by HPLC. The resulting steep water with increased propionic acid content is blended with other feed ingredients to make an animal feed containing a natural preservative. Alternatively, the steep water with increased propionic acid content is used as a feedstock for fermentations prone to fungal contamination.

Example 2. Fermentation with Endogenous Microorganisms at 48°C to Produce Propionic Acid

Same as example 1 except low phosphorous light steep water is incubated at 48oC for 24 h with agitation at 150 rpm to eliminate reducing sugars. Steep water is then cooled to 32°C and incubated at this temperature for 72 h to allow for endogenous propionic acid production. Approximately 35 g/L lactate is converted to propionic acid and other organic acids such as acetic acid and succinic acid.

Example 3. Fermentation with Propionibacteria

Same as example 1 except strains of propionibacteria, such as *Propionibacterium acidipropionici, Propionibacterium jensenii, Propionibacterium shermanii* or *Propionibacterium thoenii*, or *Propionibacterium freudenreichii*, are added exogenously to low phosphorous light steep water.

Example 4. Fermentation with Propionibacteria in Pre-Fermented Low Phosphorous Steep water

Same as example 2 except strains of propionibacteria, such as *Propionibacterium acidipropionici, Propionibacterium jensenii, Propionibacterium shermanii* or *Propionibacterium thoenii*, or *Propionibacterium freudenreichii*, are added exogenously to pre-fermented low phosphorous light steep water.

YEAST EXAMPLES

Example 1. Fermentation with Endogenous or Added Yeast

Low phosphorus steep water is incubated at 25°C for 48 hours with 2 vvm aeration with either endogenous yeasts or added yeasts such as *Candida utilis*. Approximately 10 g/L glucose, 5 g/L fructose, and 15 g/L lactate are converted to yeast biomass and CO_2. This yeast containing low phosphorus steep water is evaporated to 50% solids to produce a yeast containing corn steep liquor that is blended with other feed ingredients to make an animal feed.

Alternatively, the yeast biomass contained in low phosphorus steep water is autolyzed at pH=5.5 and 50°C for 24 hours. The resulting yeast hydrolysate containing low phosphorus steep water is evaporated to 50% solids to produce a yeast hydrolysate containing corn steep liquor that is blended with other feed ingredients for make an animal feed. The yeast hydrolysate containing corn steep liquor is also used as a nutrient source for general fermentation processes. The yeast autolysate containing low phosphorus steep water was centrifuged at 5000 x g for 15 minutes to produce a clarified yeast extract containing low phosphorus steep water that is dried to a yeast extract steep powder. The yeast extract steep powder is blended with other feed ingredients to make an animal feed or used as a nutrient source for fermentation processes.

Example 2: Fermentation with Endogenous or Added Yeast and Bisulfite

500ppm of sodium bisulfite is added to low phosphorus steep water and incubated at 35°C for 24 hours with 2 vvm aeration with either endogenous yeasts or added yeast such as *Candida krusei*. Approximately 10 g/L glucose and 5 g/L fructose are converted to yeast biomass and glycerol. The yield of glycerol is about 20%. The glycerol containing low phosphorus steep water is evaporated to 50% solids and blended with other feed ingredients to make a stable animal feed.

Numerous modifications and variations in practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing detailed description of the invention. Consequently, such modifications and variations are intended to be included within the scope of the following claims.

Claims

What is claimed is:

1. A method of making an enhanced steep water comprising:
   a) incubating the steep water under conditions that allow one or more microorganisms to grow, wherein the microorganisms alter the steep water such that it contains one or more components selected from the group consisting of bacteriocins, vitamins, yeast, yeast extracts, enzymes, amino acids, organic acids, and direct-fed microbials, at concentrations that are higher than are normally found in steep water.

2 The method of claim 1, further comprising adding exogenous microorganisms to the steep water and then incubating the steep water as described in a).

3. The product produced by the method of either claim 1 or claim 2.

Abstract of the Invention

The present invention is directed to the use of corn steep liquor which is a co-product of wet corn milling to make fermentation products which may be used in animal feeds and fermentation media.

311043

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/024015

International filing date: 26 July 2004 (26.07.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/490,349
Filing date: 24 July 2003 (24.07.2003)

Date of receipt at the International Bureau: 07 October 2004 (07.10.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)